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(57) Abstract

Vessels are treated with a mixture of L-arginine and an agent which enhances the biotransformation of L-arginine into NO. The incidents associated with restenosis are expected to be substantially reduced and prevented providing for a reduced incidence of restenosis as a result of the injury.

A THERAPEUTIC MIXTURE USEFUL IN INHIBITING LESION FORMATION AFTER VASCULAR INJURY

RELATED APPLICATION DATA

This application is a continuation-in-part application of U.S. Serial No. 09/226,580 filed January 7, 1999, which is a continuation-in-part application of U.S. Serial No. 09/833,842 filed April 10, 1997, which is a continuation-in-part application of U.S. Serial No. 08/693,882 filed August 5, 1996, now U.S. Patent No. 5,767,160 dated August 6, 1996, which is a continuation-in-part application of U.S. Serial No. 08/321,051 filed October 5, 1994, now U.S. Patent No. 5,543,430 dated June 16, 1998.

BACKGROUND OF THE INVENTION

Recently it has been established that a family of enzymes called Nitric Oxide Synthase ("NOS") form nitric oxide from L-arginine, and the nitric oxide produced is responsible for the endothelium dependent relaxation and activation of soluble guanylate cyclase, neurotransmission in the central and peripheral nervous systems, and activated macrophage cytotoxicity.

Nitric Oxide Synthase, occurs in many distinct isoforms which include a constitutive form (cNOS) and an inducible form (iNOS). The constitutive form is present in normal endothelial cells, neurons and some other tissues. Formation of nitric oxide by the constitutive form in endothelial cells is thought to play an important role in normal blood pressure regulation, prevention of endothelial dysfunction such as hyperlipodemia, arteriosclerosis, thrombosis, and restenosis. The inducible form of nitric oxide synthase has been found to be present in activated macrophages and is induced in vascular smooth muscle cells, for example, by various cytokines and/or microbial products.

because high concentrations of NO produced by iNOS can be toxic to cells. Induction of iNOS can be inhibited-by glucocorticoids and some cytokines. Relatively little is known about postranscriptional regulation of iNOS. Cytotoxic effects of NO are probably largely independent of guanylate cyclase and cyclic GMP formation.

It is known that administration of drugs consisting of nitric oxide, or releasing nitric oxide, can inhibit restenosis after angioplasty. Chronic inhalation of nitric oxide inhibits restenosis following balloon-induced vascular injury of the rat carotid artery. Oral administration of NO donors (drugs which release nitric oxide) inhibits restenosis in rat and pig models of balloon angioplasty-induced vascular injury.

The long term benefit of coronary balloon angioplasty and atherectomy is limited by the considerably high occurrence of symptomatic restenosis (40-50%) 3 to 6 months following the procedure. Restenosis is in part due to myointimal hyperplasia, a process that narrows the vessel lumen and which is characterized by vascular smooth muscle cell migration and proliferation. Medical therapies to prevent restenosis have been uniformly unsuccessful. Intravascular stents have been successfully used to achieve optimal lumen gain, and to prevent significant remodeling. However, intimal thickening still plays a significant role in stent restenosis.

The vascular architecture is maintained or remodeled in response to the changes in the balance of paracrine factors. One of the substances that participates in vascular homeostasis is endothelium derived nitric oxide (NO). NO is synthesized from the amino acid L-arginine by NO synthase. NO relaxes vascular smooth muscle and inhibits its proliferation. In addition, NO inhibits the interaction of circulating blood elements with the vessel wall. NO activity is reduced in hypercholesterolemia and after vascular injury. The administration of L-arginine alone has been shown to restore vascular NO activity in animals and in humans with endothelial vasodilator dysfunction.

above which results in pre-determined amounts of a NOS agonist and a NOS substrate.

"Agonist" refers to an agent which stimulates the bio-transformation of a NO precursor, such as L-arginine or L-lysine to EDNO or EDRF either through enzymatic activation, regulation or increasing gene expression (*i.e.*, increased protein levels of c-NOS). Of course, either or both of these mechanisms may be acting simultaneously.

As used herein, the term "pharmaceutically acceptable carrier" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not toxic to the hosts to which it is administered.

Methods and devices are provided for inhibiting the pathology associated with vascular injury, particularly during angioplasty and atherectomy. A NO producing mixture, preferably L-arginine and a NOS agonist, is introduced either intraluminally or more preferably intramurally (for example by a stent) to into the walls of the injured vessel in proximity to the injury in an amount to inhibit the pathology, *e.g.*, restenosis, associated with the vascular injury. Various conventional delivery devices may be used for the delivery of the therapeutic mixture.

The following examples or embodiments are offered by illustration, and not by way of limitation.

The present invention is generally directed to treating vessels with a mixture of L-arginine and an agent which enhances the biotransformation of L-arginine into NO. The incidents associated with restenosis are expected to substantially reduced and prevented providing for a reduced incidence of restenosis.

biological equivalent of L-arginine into nitric oxide. It is preferable that the method further includes the step of introducing an agent which prevents the degradation of said nitric oxide. In this embodiment the step introducing may be by means of a local delivery catheter.

In an alternative embodiment there is provided a method for reducing the probability of restenosis resulting from injury caused by angioplasty or atherectomy, comprising: introducing intramurally or intraluminally, preferably intramurally at the site of said injury a stent, said stent having a body comprised of L-arginine and a NOS agonist.

An alternative embodiment of the present invention provides a stent having a body comprising a NO precursor agent and a NOS agonist, the NO precursor includes at least one of L-arginine or L-lysine, and the a NO precursor agent and NOS agonist releasable under conditions present in a blood vessel.

An alternative embodiment of the present invention provides a stent having a body comprised of L-arginine and a nitrate, preferably nitroglycerin.

An alternative embodiment of the present invention provides a stent having a body comprised of L-arginine and an Hmg-CoA reductase inhibitor, preferably atorvastatin or pravastatin.

An alternative embodiment of the present invention provides a stent having a body comprised of L-arginine and an angiogenic growth factor.

An alternative embodiment of the present invention provides a stent having a body comprised of L-arginine and DOX.

An alternative embodiment of the present invention provides an anti-restenosis device comprised of a body, said body including a therapeutic

In this embodiment the NO precursor may be L-lysine. In this embodiment the NO precursor agent may be an arginase inhibitor. In this embodiment the agent which enhances the conversion of the precursor agent to native NO may be a nitrate. In this embodiment the agent which enhances the conversion of the precursor agent to native NO may be nitroglycerin. In this embodiment the agent which enhances the conversion of the precursor agent to native NO may be an Hmg-CoA reductase inhibitor. In this embodiment the agent which enhances the conversion of the precursor agent to native NO may be a statin. In this embodiment the agent which enhances the conversion of the precursor agent to native NO may be pravastatin. In this embodiment the agent which enhances the conversion of the precursor agent to native NO may be an antiogenic growth factor. In this embodiment the agent which enhances the conversion of the precursor agent to native NO maybe DOX.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A is the top portion of a schematic representation of proposed L-arginine dependent and independent pathways.

Fig. 1B is the bottom portion flowing from Fig. 1A of a schematic representation of the proposed L-arginine dependent and independent pathways.

Fig. 2 is a bar graph illustrating the NOS stimulating effect of combined administration of L-arginine and nitroglycerin on rat aorta.

Fig. 3 is a schematic representation of the proposed NOS activation pathway involving pravastatin.

Fig. 4 is a bar graph illustrating the stimulation of NOS with pravastatin.

Fig. 15 indicates the effect of s-nitroso-acetyl-penicillamin (SNAP, 200 μM; equivalent to 0.4 μM NO) on y^+ transport of [^{3}H]-LA in bovine aortic endothelial cells.

Fig. 16 indicates the effect of dipropylenetriamine NONOate (DPTA, 10-0.01 μM; equivalent to 20-0.02 μM NO) on y^+ transport of [^{3}H]-LA in bovine aortic endothelial cells.

Fig. 17 indicates the effect of L-arginine (LA, 5 x 10^{-4}M) and n-ω-nitro-L-arginine methyl ester (L-NAME, 5 x 10^{-4}M) on substance P (SP, 1 μM) or calcium ionaphore, A-23187 (CI, 1 μM) induced superoxide anion ($O_2^{\bullet-}$) formation in bovine aortic endothelial cells (BAEC).

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Devices and methods are provided for the treatment of pathologies associated with vascular injury, particularly in relation to angioplasty and atherectomy. Of particular interest is the injury referred to as restenosis, which results from the migration and proliferation of vascular smooth muscle cells into the intima of the vessel as well as accretions associated with the atherosclerosis.

The method provides introducing to or into the vessel walls at the site of injury a therapeutic mixture which includes at least a NO precursor and more preferably a combination of a NO precursor and an agent which enhances the conversion of the NO precursor to NO and which results in the enhancement of NO production in the cells at the site of injury. Various delivery systems may be employed which result in the therapeutic mixture infusing into the vessel wall, and being available to the cells for NO production. Devices which may be employed include drug delivery balloons, *e.g.*, porous, sonophoretic, and iontophoretic balloons, as exemplified by the devices depicted in WO92/11895, WO95/05866 and WO96/08286, which are incorporated herein by reference thereto. Also, stents may be employed

The subject methodology is employed with hosts who have suffered vascular injury, as caused by angioplasty and atherectomies. The time for the administration of the therapeutic mixture may be varied widely, providing a single administration or multiple administrations over a relatively short time period in relation to the time of injury. Generally, treatment may be before, concurrently or after the injury, usually within 2 weeks of the injury, if before, and not more than about 8 weeks, usually not more than about 6 weeks, preferably in the range of 0-6 weeks (where 0 intends concurrently or shortly after the prior procedure, within 6 hours).

It is expected that with one treatment of the NO precursor agent at or about the time of the injury, before or shortly thereafter, one will observe enhanced vascular NO production and reduced intimal thickening, so as to substantially reduce the potential for restenosis.

In conjunction with the intraluminal or intramural deliver of the therapeutic mixture by the catheter, a stent may be introduced at the site of vascular injury. The stent may be biodegradable or non-biodegradable, may be prepared from various materials, such as metals, ceramics, plastics or combinations thereof. Biodegradable plastics, such as polyesters of hydroxycarboxylic acids, are of particular interest. Numerous stents have been reported in the literature and have found commercial acceptance. An example of the type of stent which may be modified to deliver an arginine based mixture (including an agent which enhances production of NO) is shown in U.S. Pat. Nos. 5,665,077, 5,482,925, and 5,405,919, each of which are incorporated by reference hereto in their entirety.

Depending on the nature of the stent, the stent may have the therapeutic mixture incorporated in the body of the stent or coated thereon. For incorporation, normally a biodegradable plastic stent will be used which will release the therapeutic mixture while supporting the vessel and protecting against restenosis. In the fabrication of the stent, the biodegradable matrix may be formed by any convenient means known in the

entirety. The following discussion will focus on smooth muscle and myocyte relaxation stimulated by nitrovasodilators wherein the nitric oxide synthase is cNOS, the constitutive form of nitric oxide synthase, the generator cells are endothelial cells and the target cells are vascular smooth muscle cells. This illustration is not intended to imply any cellular relationship between the various sites of action, but rather meant to illustrate their functional relationship.

As shown in Figs. 1A and 1B the production of NO may result from a variety of sources and mechanisms which are discussed in detail in Ignarro, (Louis J. PhD., 1991, Pharmacology of Endothelium-Derived Nitric Oxide and Nitrovasodilators, The Western Journal of Medicine, pp.51-62.). In the L-arginine independent or non-endothelium dependent pathway the activation of Guanylate Cyclase (GC) by Nitric Oxide (NO) depends on the type of nitrovasodilator used. Inorganic Nitrite (NO_2^-) is charged and only limited amounts can permeate the cell, but intracellular nitrite can be converted to NO. Lipophilic organic nitrate esters (R--OH) are converted into NO by acidic thiol (R--SH) facilitated reactions. S-Nitrosothiols (R--SNO) are labile intermediates that decompose spontaneously and produce NO. It is thought that one of the mechanisms by which thiols potentiate the action of nitroglycerin and reverse to some degree tolerance to nitroglycerin is through the direct reaction between the thiol (R--SH) and nitroglycerin (GTN) to form the labile intermediate S-Nitrosothiol (R--SNO), which decompose as described above (R--SH+GTN -->R--SNO is not shown). A nonenzymatic formation of exogenous NO is thought to occur with thiol sources such as cysteine, dithiothreitol, N-acetylcysteine, mercaptosuccinic acid, thiosalicylic acid, and methylthiosalicylic acid.

It is hypothesized that the tolerance to nitroglycerin may involve a secondary pathway, or indeed, this "secondary pathway" may be the primary pathway. This "secondary pathway" is the L-arginine dependent pathway or endothelium dependent pathway shown in Figs. 1A and 1B. As

principally a venodilator at low doses although it can become a veno-arterial dilator at high doses and causes rapid increase in heart beat due to its venous pooling, while L-arginine on the other hand when used alone is principally an arterial dilator. Therefore, combining the two results in balanced arterial and venodilatory effect which counter balances the tendencies of one or the other to produce tachycardia which is adverse to ischemia in an evolving myocardial infarction.

Another mechanism of benefit from the combination relates to the fact that used alone nitroglycerin is of only minimal benefit in limiting reperfusion injury with patients who have had recent heart attacks and abrupt restoration of blood flow. The same thing is seen in patients who are undergoing re-establishment of blood flow after coronary bypass operations coming off the bypass pump.

We discovered that dogs treated to a floor of nitroglycerin effect could be made further responsive by the co-administration of nitroglycerin and L-arginine in water in a manner similar to that commonly seen clinically with the addition of sodium nitroprusside (SNP) to nitroglycerin; however, when compared to SNP, L-arginine combined with nitroglycerin had much more favorable hemodynamic effects. Compared to SNP, vascular resistance was reduced by 50%, cardiac output doubled, and contractility increased. This led to the hypothesis that the combination of L-arginine and nitroglycerine was generating EDRF as opposed to SNP which is known to produce nitric oxide in a direct fashion. The following key corresponds to the bar graph shown in Fig. 2.

A. Control--Basal. This represents cGMP activity at baseline that was generated by resting NO sources of soluble guanylate cyclase activation, *i.e.*, baseline.

B. L-arginine Group. This represents cGMP activity generated by L-arginine and EDRF (endogenous or "native" NO production).

activation site is cNOS in the endothelial cell. Under conditions leading to tolerance the agonist effect of nitroglycerin on cNOS induction leads to a depletion of L-arginine in the endothelial cell secondary to rate limitations in active L-arginine transport pump kinetics in Fig. 1A and Fig. 1B. This creates a supply demand mismatch situation at the membrane uptake step and explains why arginine is rate limiting. This may also explain why during administration of nitroglycerin a nitrate free interval is required. It is believed that this is necessary so that the endothelial cells can replete the deficient L-arginine by active transport. By adding L-arginine when administering nitroglycerin it is believed that EDRF can be generated, and in the process a significant reduction in clinical and mortality endpoints can be obtained relative to using nitroglycerin alone or in combination with SNP or other donors of exogenous NO.

It has been shown that nitroglycerin applied at the site of intimal injury following balloon angioplasty reduces the formation of medical cellular proliferation. However, intimal and neointimal proliferation were not reduced. This was thought to be secondary to the development of tolerance to nitroglycerin. We have shown that tolerance to nitroglycerin may in fact be related to its function as a NOS agonist. The activation of Nitric Oxide Synthase which results in a development of tolerance to the effectiveness of the nitroglycerin and the fact that tolerance to nitroglycerin can be overcome by the concomitant administration locally of L-Arginine, its salts or of its biological equivalents, such as Lysine provide a heretofore unexpected benefit of the application of a mixture of nitroglycerin and L-arginineutilized an acute or chronic intrarterial site-specific locally indwelling and/or elating anterestenosis drug delivery device at the site of balloon injury.

In another embodiment of the invention, therapeutically effective amounts of L-arginine and inhibitors of Hmg-CoA reductase are mixed at a physiologically acceptable pH and administered to a patient.

ratios of an Hmg-CoA reductase inhibitor to L-arginine may be employed with virtually any Hmg-CoA reductase inhibitor.

Where the particular Hmg-CoA reductase inhibitor is pravastatin, the ratio of pravastatin to L-arginine is preferably within the range 1:2 to 1:50, Wt/Wt. For example, pravastatin/L-arginine at a ratio of 1:2 would include 40 mg/day pravastatin with 80 mg/day L-arginine. Where the ratio of pravastatin/ L-arginine is at a ratio of 1:20, for example, 20 mg/day pravastatin would be administered with 400 mg/day L-arginine. Weight ratio of ingredients described herein in regard to the Hmg-CoA reductase inhibitors, lovastatin, pravastatin and atorvastatin are applicable for any Hmg-CoA reductase inhibitor. The amounts above have been found to be effective, however, each route of administration (*e.g.*, IV, oral, transdermal, etc.) will vary in their requirements.

Even more particularly, the presently disclosed "mixtures" may be described in terms of their relative concentrations (grams) administered as part of a continuous daily and/or monthly regimen. In one particular embodiment, the formulation is administered so as to provide the patient with between 20-40 milligrams per day of the Hmg-CoA reductase inhibitor (*e.g.*, pravastatin) together with a daily dose of L-arginine of between 100 to 200 mg per day. Most preferably, the Hmg-CoA reductase inhibitor, such as lovastatin, is administered at a daily dose of about 20 mg per day together with a dose of about 200 mg per day L-arginine. This particular embodiment of the claimed formulation should maintain within the patient efficient levels of the formulation.

The Hmg-CoA reductase inhibitors of the present invention are also characterized by an ability to stimulate receptor-mediated clearance of hepatic low-density lipoproteins (LDL), as an anti-hypercholesterolemic, and as a competitive inhibitor of Hmg-CoA reductase.

To demonstrate this, the direct effects of acteylcholine and pravastatin on NO production in bovine aortic endothelial cells (BAEC) was determined using a highly sensitive photometric assay for conversion of oxyhemoglobin to methemoglobin. NO oxidize; oxyhemoglobin (HbO_2) to methemoglobin (metHb) in the following reaction HbO_2 + NO - metHb + NO_3. The amount of NO produced by endothelial cells was quantified by measuring the change in absorbance as HbO_2 oxidizes to metHb. Oxyhemoglobin has a absorbance peak at 415 nm, while metHb has a 406 nm absorbance peak. By subtracting the absorbance of metHb from HbO_2, the concentration of NO can be assessed. The general method was patterned after that of *Feelisch et al.*, (Biochem. and Biophy. Res. Comm. 1991; 180, Nc I:286-293).

Fig. 4 is a bar graph of the data generated which illustrates the effects of acetylcholine and pravastatin (10^{-6} and 10^{-5} M) administered for 3 min periods into the cell/bead perfusion system on NO production with: 1) 10^{-5} M L-arginine in control (basic) buffer, 2) 10^{-3} M of L-NAME in buffer, and 3) 10^{-3} M of L-arginine in buffer. Responses are transient elevations in NO production above basal levels. Data for responses in L-NAME and L-arginine augmented buffer are presented as percent of response in control buffer (100%); numbers in basic buffer bars indicate absolute production of NO in nmole *min. The remaining two bars denote differences between responses in L-NAME buffer vs both basic and L-arginine added buffers.

The effects of pravastatin on activity of endothelial cells in producing NO were compared with those of actetylcholine, which is known to specifically stimulate NO production by NOS activity. Adding acetylcholine to the buffer superfusion bovine aortic endothelial cells (BAECs) grown on beads increased their production of NO as measured by oxidation of oxyhemoglobin to methemoglobin. Acetylcholine produced a transient, concentration-related increase in NO above baseline levels. In basic buffer containing 5 x 10^{-5}M L-arginine, and there was approximately a two fold

possible angiogenic factor. Angiogenic growth factors like those identified in Table I stimulate the growth of new blood vessels (*e.g.*, in vascular beds such as the coronary, peripheral, etc.) previously occluded with atherosclerotic obstructions. Angiogenic growth factors are proteins which were initially discovered as agents responsible for the growth of new blood vessels which maintain the growth and spread of cancerous tumors (neovascularization). Two of the angiogenic growth factors, vascular endothelial growth factor (VEGF) and basic fibroblastic growth factor (bFGF), have been infused into catheters, used at the time of generating coronary and peripheral arteriograms, and have resulted in the growth of significant new collateral blood vessels in the region of ischemia producing vascular atherosclerotic occlusions. In this way, the symptoms of ischemia are lessened. The term applied to this treatment approach is "therapeutic angiogenesis."

Like angiogenic agents Substance P and Bradykinin, VEGF and bFGF also appear to act as NOS agonists, specifically cNOS. It appears the resultant EDNO produced is in large part responsible for the new collateral vessel growth ("collateral") which in turn is responsible for the improvement in symptoms of ischemia seen in therapeutic angiogenesis. Furthermore, it has also been shown that the collateral responses to both VEGF and bFGF can be magnified significantly with L-arginine supplementation. Therefore, angiogenic growth factors, preferably VEGF and bFGF, appear to have dual applicability in the treatment of hypertension and cardiovascular diseases in that they both stimulate therapeutic angiogenesis and activity of Nitric Oxide Synthase. It also appears that the overall therapeutic angiogenic result with angiogenic growth factors is augmented to the extent they act as agonists of NOS. The fact that angiogenic growth factors are agonists or stimulators of nitric oxide synthase has important implications. Mixing angiogenic growth factors "in vitro" or "in vivo" with L-arginine may have an unforeseen beneficial effect that is associated with excess L-arginine providing additional substrate for NOS and the NOS being catalyzed to

Where the particular angiogenic growth factor is VEGF the ratio of VEGF to L-arginine is preferably within the range 1:2 to 1:50, Wt/Wt. For example, VEGF/L-arginine at a ratio of 1:2 would include 40 mg/day VEGF with 80 mg/day L-arginine. Where the ratio of VEGF/ L-arginine is at a ratio of 1:20, for example, 20 mg/day VEGF would be administered with 400 mg/day L-arginine. Weight ratio of ingredients described herein in regard to VEGF or bFGF are generally applicable. The amounts above have been found to be effective, however, each route of administration (*i.e.*, IV, oral, transdermal, intracoronary, intra-arterial, etc.) may vary in their requirements.

Fig. 5 is a schematic illustration of a proposed mechanism of action of preferred substances (*e.g.*, angiogenic growth factors) and arginine and is not intended to imply any cellular relationship or geography of the various sites of action, but rather meant to illustrate their functional relationship. Fig. 5 lists certain preferred agents as angiogenic agents and is meant as a representative sampling. SP represents Substance P and GF representing select Growth Factors.

As indicated, various delivery devices may be employed for the delivery of the active agent(s). Fig. 6 illustrates the drug delivery apparatus with the balloon 12 in its inflated state and within an arterial vessel in which the vessel walls are indicated by the reference numeral 15. During percutaneous transluminal coronary angioplasty ("PCTA") procedures, the guide wire 10 is first inserted into the selected artery to a point past the stenotic lesion. The dilatation catheter including the catheter body 11 and the balloon 12 is then advanced along the guide wire 10 to the desired position in the arterial system in which the balloon portion 12 traverses or crosses the stenotic lesion. The balloon 12 is then inflated by introducing the solution containing the therapeutic mixture (or the NO precursor if subsequent or simultaneous delivery of a second agent is being employed) through the balloon lumen 14 into the interior chamber 13 of the balloon 12.

agent(s) across the balloon wall 26 and into contact with the vessel walls 15. One electrode 28, the catheter electrode, is located on or within the catheter body 11, while the other electrode 31, the body surface electrode, is located on the body surface or within the body of the patient. An electrical current for the iontophoretic process is produced between the electrodes 28 and 31 by an external power source 30 through the electrical leads 29 and 33, respectively. Direct current may be used, although other wave forms are also utilized (*e.g.*, a series of rectangular waves producing a frequency of 100 Hz or greater).

During operation of the iontophoretic device, the balloon 26 is first positioned across the stenotic lesion. The balloon interior 27 is then inflated with the drug in the lumen 23. As the balloon expands, it causes the artery to dilate. This is followed by activating the power supply 30, thereby creating a current between the electrode 28 and the electrode 31 which passes through the balloon wall 26. This current drives or drags the agent(s) (*e.g.*, NO precursor and nitroglycerin) within the chamber 27 across the wall and into contact with the surrounding vessel wall 15 and vascular tissue.

In Fig. 8 is shown a device 30 comprising a catheter 32 carrying a mesh stent 34 encircling balloon 36 in its collapsed state. The mesh stent 34 would be covered for example with a slow release layer of L-arginine/NOS agonist or L-arginine/statin (e.g., pravastatin) containing poly(glycolide-lactide) 38. The coronary artery vessel 40 is shown with the lesion partially closing the coronary vessel artery. In Fig. 10, the balloon 42 has been expanded so as to expand stent 44 to press against the vessel wall 46 and open the vessel lumen 48. The coating 50 on the stent 44 can now release the active agent(s) directly into the vessel wall to inhibit vascular smooth muscle proliferation.

The stent is introduced into the appropriate position as previously described for directing the balloon for angioplasty. However, in this case, the balloon is surrounded by the stent. As indicated above, when the balloon and stent are appropriately positioned, the balloon is expanded

Pharmaceutically-acceptable carriers may also be comprised of excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, PA) hereby incorporated herein by reference in its entirety. The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of administration.

Treatment with L-arginine or with other agents that increase eNOS activity and NO production have been found to protect against vascular injury in various experimental models. Since these treatments also stimulate tPA (tissue plasminogen activator) production and/or inhibit production of PAI-1, it is likely that their protective effects are due at least in part to effects in increasing tPA activity. Recently, many agents whose principal actions are unrelated to eNOS activity have been shown to have independent auxiliary actions through eNOS activation and NO production. These include organic nitrates, converting enzyme inhibitors, amrinone, nevilolol, S-nitroso-TPA, pravastatin and amlodipine. Doxazosin appears to have a similar auxiliary mechanism and that it increases TPA levels as a result of effects on eNOS activation in ECs.

Nitric Oxide Measurements. Methemoglobin - The effect of DOX on NO production in EC was determined using a photometric assay for conversion of oxyhemoglobin to methemoglobin. For this assay, EC grown to confluency on microcarrier beads are placed into a water-jacketed chromatography column and superfused with a Kreb's-Ringer buffer containing 3 μM oxyhemoglobin and 50 μM LA (L-arginine). Perfusate is then directed into a flow-through cuvette in a dual wavelength spectrophotometer and change in absorbency (415/405 nm) is measured. Experimental stimulation was carried out by 3 min infusion periods of DOX added to buffer perfusion to yield final concentrations of 10^{-7} and 10^{-6} M. For analysis, we determined the area under the curve for the change in absorbency response/min caused by DOX assuming a one to one correspondence for NO and metHb production, the known stoichiometric balance for this reaction. NO production was measured with a NO meter connected to a polargraphic NO electrode as previously described. The NO sensor probe will be inserted vertically into 24-well plates containing confluent EC such that the tip of the electrode is submerged 2 mm under the surface of medium (above - 1ml). The reaction was initiated when desired concentrations of DOX are added to the well. Calibrations were performed with S-nitroso-acetyl-penicillamine.

TPA assay. In the TPA assay, EC was grown to confluency in 24-well plates and on experimental days, the medium was discarded and replaced with 0.5ml serum-free M199 containing 1% BSA and 50μM LA and incubated at 37% for 48 hrs in the presence of DOX (10^{-7} to 10^{-5} M) or acetylcholine (10^{-7} and 10^{-6} M) with and without L-NAME and excess LA. After incubation, the medium was harvested for determination of tPA content by an ELISA kit.

It would appear that DOX, like nitroglycerin, substance P and bradykinin, acts as a NOS agonist. It appears that the responses to DOX can be magnified significantly with L-arginine supplementation. It appears the

factor following transfection of the genetic material into the surrounding tissue of the vascular bed. In some cases pellets containing the aforementioned mixtures may be directly implanted into the myocardium at the time of coronary bypass graft surgery. In yet another case, a therapeutic mixture of L-arginine and DOX may be repeatedly infused into the pericardial space via an indwelling infusion catheter.

The therapeutically effective dose of DOX can be estimated initially either in cell culture assays, *e.g.*, of neoplastic cells, or in animal models, usually mice, rabbits, dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy.

An alternative embodiment of the present invention is based on a the fact that when cellular supply of L-arginine is limited, NOS utilizes molecular oxygen as a lone substrate producing superoxide anion ($O_2^{\bullet-}$) and other reactive free radicals which can lead to cardiovascular dysfunction and the pathogenesis of disease.

The total intracellular concentration of L-arginine (0.1 - 1mM) in endothelial cells (EC) greatly exceeds the K_m of eNOS for L-arginine (~3 μM). This suggests that eNOS is saturated with substrate and that levels of intracellular L-arginine are not limiting for NO production. However, other

of L-arginine into EC occurs through two carrier-mediated transporters and passive diffusion. The saturable carrier-mediated transporters include a sodium-dependent active transporter, system B^{0+} and a sodium-dependent transporter, system y^+. The majority (80%) of L-arginine delivered into most cells is through the y^+ transporter. Regulation of L-arginine transport appears to involve cellular membrane potential. Exposure of endothelial cells to hyperpolarizing agents including ATP and bradykinin increases L-arginine uptake while a decrease in L-arginine transport was observed when cells were treated with agents that cause cellular depolarization. In addition, factors that reduce the activity of the y^+ transporter, including free radicals, may also reduce L-arginine available for NOS.

When the balance of transporter regulatory factors is negative, L-arginine supply becomes limiting and subsequent production of $O_2^{\bullet-}$ may contribute to vascular and organ pathology. We compared the effects of NOS agonists and NO donors on L-arginine uptake by EC. Effects of NOS stimulation on superoxide anion production were also assessed in the presence and absence of L-arginine and the NOS antagonist, L-NAME.

Fig. 10 is a schematic representation of the hypothesized dynamics of L-arginine supply to NOS. L-arginine levels are maintained primarily through the activity of the carrier-mediated Na^+-independent transporter, y^+, while the Na^+-dependent transporter, B^{0+}, and passive diffusion account for less than 15%. Concurrent transport of L-arginine to NOS may control NO production. However, L-arginine supply to NOS can be limiting due to compartmentalization within EC, arginase activity or utilization of L-arginine by NOS. We believe that NO and superoxide anion reduce the activity of the y^+ transporter and also reduce L-arginine available for NOS. Collectively, summation of supply verses demand or availability of L-arginine to NOS will determine whether NO or superoxide anion are formed.

Fig. 13 indicates the effect substance P (SP, 1 μM) on y^+ transport of [^{3}H]-LA in bovine aortic endothelial cells. Cells were exposed to SP and incubated with sodium-free uptake buffer containing tritiated L-arginine and the amount of [^{3}H]-LA delivered to cells was determined as described in "Methods." Data are presented as mean S.E.M; $p<0.05$ from control values. As can be seen in Fig. 13, substance P (SP) was also effective in augmenting cellular uptake of L-arginine into cells. SP increased y^+ transport of L-arginine into cells by 24% after only 15 minutes exposure. This elevated L-arginine uptake was maintained for exposures of 30 and 60 minutes with 24 and 21% increases, respectively. In addition, the effect of SP on cellular transport of [^{3}H]-LA was enhanced after pre-treatment with SP for more prolonged durations. After 2 hour exposure of BAEC to SP, y^+ transporter activity was enhanced as much as 34% from control values. This increase in transporter activity was also maintained after 3 and 5 hour exposure with cellular L-arginine increases of 27 and 21%, respectively.

Effects of a third NOS agonist, acetylcholine (Ach) on the cellular uptake of [^{3}H]-LA are shown in Fig. 14. Fig. 14 indicates the effect of acetylcholine (Ach, 5 μM) on y^+ transport of [^{3}H]-LA in bovine aortic endothelial cells. Cells were exposed to Ach and incubated with sodium-free uptake buffer containing tritiated L-arginine and the amount of [^{3}H]-LA delivered to cells was determined as described in "Methods." Data are presented as mean S.E.M; $p<0.05$ from control values. Incubation with Ach increased L-arginine transport over all time periods. A 22% increase of [^{3}H]-LA uptake was observed after 2 minute exposure to Ach. After 15 minute addition of Ach, L-arginine uptake reached to a maximum increase of 27%. Treatment with Ach for 30 or 60 minutes resulted in 16 and 15.5% increases of L-arginine uptake, respectively.

Effect of NO donors on cellular uptake of LA. Fig. 15 indicates the effect of s-nitroso-acetyl-penicillamin (SNAP, 200 μM; equivalent to 0.4 μM NO) on y^+ transport of [^{3}H]-LA in bovine aortic endothelial cells. Cells

anion formation, experiments were performed in which cellular production of superoxide anion was monitored alone (basal) and during treatment with SP (1 μM) or the calcium ionophore A-23187 (1 μM), with or without concurrent presence of L-arginine or L-NAME supplementation. Fig. 17 indicates the effect of L-arginine (LA, 5 x 10^{-4}M) and n-ω-nitro-L-arginine methyl ester (L-NAME, 5 x 10^{-4}M) on substance P (SP, 1 μM) or calcium ionaphore, A-23187 (CI, 1 μM) induced superoxide anion ($O_2^{\bullet-}$) formation in bovine aortic endothelial cells (BAEC). BAEC were treated with SP or A-23187 in the presence or absence of L-arginine or L-NAME and $O_2^{\bullet-}$ production was determined over a 60 minute time period and compared to basal levels as described in "Methods." Data are presented as mean S.E.M; $p<0.05$ from control values. Fig. 9 demonstrates that $O_2^{\bullet-}$ is produced by BAEC and that supplementation with L-NAME, but not LA, prevented basal production of $O_2^{\bullet-}$ by 100%. Addition of SP or A-23187 significantly increased $O_2^{\bullet-}$ production above basal levels by 3.5 and 2.5 fold, respectively. Concurrent treatment with either L-arginine (5 x 10^{-4}M) or L-NAME (5 x 10^{-4}M) effectively reduced $O_2^{\bullet-}$ induced by SP by 51 and 81%, respectively. Similar inhibitory effects of L-arginine and L-NAME on $O_2^{\bullet-}$ production were observed when the calcium ionophore A-23187 was used to induce NOS activation, with 60 and 58% inhibition observed with L-arginine and L-NAME, respectively.

The transport of L-arginine to cells is critical for maintaining adequate L-arginine levels such that optimal coupling of L-arginine with endothelial NOS (eNOS) can occur. Therefore, factors affecting the y^+ transporter system have the potential to limit the production of NO. Without ample LA, eNOS will solely utilize O_2 to form $O_2^{\bullet-}$ that may contribute to the pathogenesis of disease. As a consequence, controlling L-arginine supply and other factors affecting superoxide production would be beneficial in normal as well as pathological circumstances.

of L-arginine is expected as NO is known to cause cellular hyperpolarization. However, longer exposures of 1 to 4 hours resulted in a marked reduction of L-arginine transport. These data were confirmed by using a different NO donor, DPTA, to stimulate prolonged exposure of cells to NO. DPTA releases NO slowly over time and, therefore, was used to repeat the longer durations of NO exposure. Although one might expect to see a continued increase of y^+ transporter activity with NO exposure similar to that observed using NOS agonists, there is evidence that oxidative properties of NO may be responsible for the reduction of cellular L-arginine transport seen with longer exposure periods. It has been demonstrated that NO, through constant gas infusion and release from SNAP, decreases y^+ system transporter activity. The negative effect of NO on y^+ transport of L-arginine into cells was determined to be associated with oxidation of sulfhydryl moieties in the transporter proteins since treatment with disulfide reducing agent dithiothreitol restored transporter activity. Furthermore, treatment of endothelial cells with sulfhydryl reactive chemicals N-ethylmaleimide (NEM) and acrolein reduced y^+ transporter activity. Collectively, these data suggest that the effects of NO on cellular y^+ L-arginine transport activity are two-fold. The initial effect seen upon acute exposure is more likely due to the hyperpolarizing properties of NO while the latter inhibitory effects observed with more prolonged exposure to NO may be the result of a summation of cell hyperpolarizing and transport oxidizing properties of NO, the latter becoming more predominant.

The biphasic effect in transport function over time noted for SNAP was not observed in cells treated with prolonged exposure to NOS agonists. It would be expected that stimulation of NOS would also increase NO production and oxidation of the y^+ transporter system resulting in inhibition of L-arginine uptake similar to that observed with SNAP. One explanation for lack of biphasic action with NOS agonists could be that the amount of NO produced upon NOS activation is far less than the amount of NO released from SNAP. Therefore, levels of NOS derived NO never

eNOS. Stimulation of BAEC with SP or A-23187 produced amounts of $O_2^{\bullet-}$ much greater than basal levels. Interestingly, a striking reduction of $O_2^{\bullet-}$ production was observed upon extracellular addition of either L-arginine or L-NAME, following treatment with SP and A-23187. These data also suggest that excessive $O_2^{\bullet-}$ formation associated with agonist induced eNOS activation, but not basal production, can be ameliorated with L-arginine supplementation.

Collectively, our findings strongly suggest that although intracellular L-arginine levels far exceed the concentration of L-arginine required by NOS for NO production, the amount of L-arginine available for utilization by NOS can be insufficient especially in conditions of chronic eNOS stimulation. The explanation for this L-arginine paradox may be provided by the work of McDonald and colleagues. Using porcine pulmonary artery endothelial cells with antibodies specific for caveolin, eNOS and the y^+ transporter, McDonald *et al.* demonstrated that all of these proteins are co-localized within the plasma membrane caveolae. This suggests that eNOS associated with this complex is sequestered from overall intracellular L-arginine and relies on the *de novo* transport of L-arginine into the cell via the y^+ transporter within the caveolae for NO production. If the transporter becomes damages as seen with oxidation, L-arginine supply could immediately become limiting and may be the basis for endothelial dysfunction. In addition, this eNOS/y^+ transporter-caveolae complex may explain why endothelial dysfunction is quickly reversed with increasing extracellular LA. Once the transporter is turned off, L-arginine concentration gradient increases and delivery of L-arginine into cells is shifted towards passive diffusion. Therefore, extracellular supplementation of L-arginine may be helpful in driving passive diffusion of L-arginine when the integrity of carrier-mediated transporters cannot be maintained.

We believe that concurrent L-arginine supply to NOS via system y^+, independent of overall intracellular L-arginine, is critical in

What is claimed is:

1. A method for reducing the probability of restenosis, said method comprising:

introducing intramurally to a site of an injury, a therapeutic mixture, said therapeutic mixture including a biological equivalent of L-arginine, and an agent which enhances NO availability.

2. The method of claim 1, wherein said agent is a NOS agonist.

3. The method of claim 2, wherein said agent is a nitrate.

4. The method of claim 3, wherein said nitrate is nitroglycerin.

5. The method of claim 1, wherein said biological equivalent of L-arginine is selected from the group consisting of L-arginine, L-lysine and a combination of L-arginine and L-lysine.

6. A method for reducing the probability of restenosis resulting from vascular injury, said method comprising:

introducing intramurally proximal to the site of said injury over a predetermined time of an active agent, said active agent including a nitric oxide precursor and an Hmg-CoA reductase inhibitor.

7. The method of claim 6, wherein said nitric oxide precursor is a biological equivalent of L-arginine.

8. A method for reducing the probability of restenosis resulting from injury caused by angioplasty or atherectomy, said method comprising:

17. The drug delivery device of claim 12, wherein the agent which enhances the conversion of the precursor agent to native NO is nitroglycerin.

18. The drug delivery device of claim 12, wherein the agent which enhances the conversion of the precursor agent to native NO is an Hmg-CoA reductase inhibitor.

19. The drug delivery device of claim 12, wherein the agent which enhances the conversion of the precursor agent to native NO is a statin.

20. The drug delivery device of claim 12, wherein the agent which enhances the conversion of the precursor agent to native NO is pravastatin.

21. The drug delivery device of claim 12, wherein the agent which enhances the conversion of the precursor agent to native NO is an antiogenic growth factor.

22. The drug delivery device of claim 12, wherein the agent which enhances the conversion of the precursor agent to native NO is DOX.

FIGURE 1A

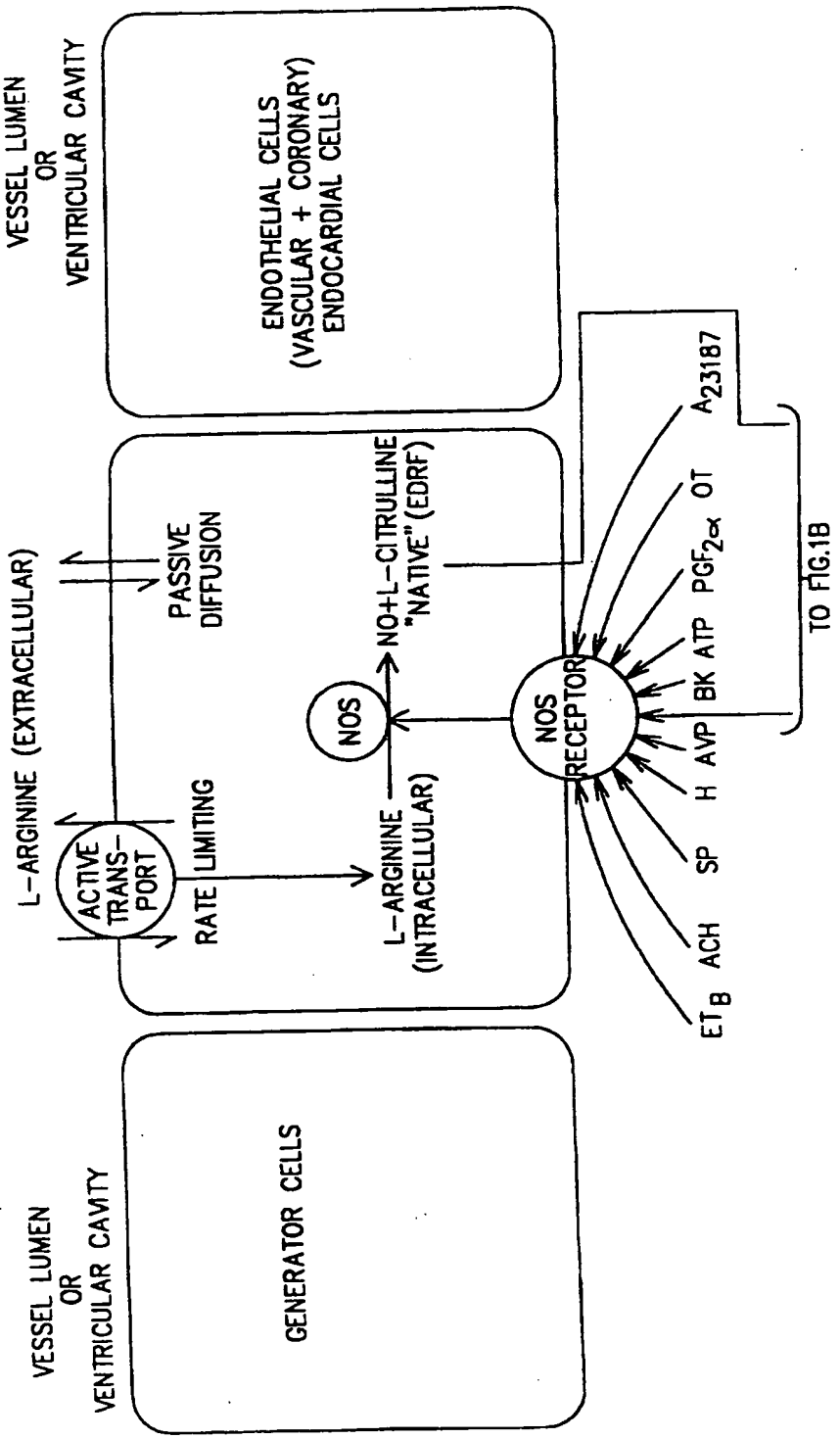

VESSEL LUMEN OR VENTRICULAR CAVITY
ENDOTHELIAL CELLS (VASCULAR + CORONARY) ENDOCARDIAL CELLS
L-ARGININE (EXTRACELLULAR)
ACTIVE TRANS-PORT
RATE LIMITING
PASSIVE DIFFUSION
NOS
L-ARGININE (INTRACELLULAR)
NO+L-CITRULLINE "NATIVE" (EDRF)
NOS RECEPTOR
ET_B ACH SP H AVP BK ATP $PGF_{2\alpha}$ OT A_{23187}
TO FIG.1B
VESSEL LUMEN OR VENTRICULAR CAVITY
GENERATOR CELLS

FIGURE 1B

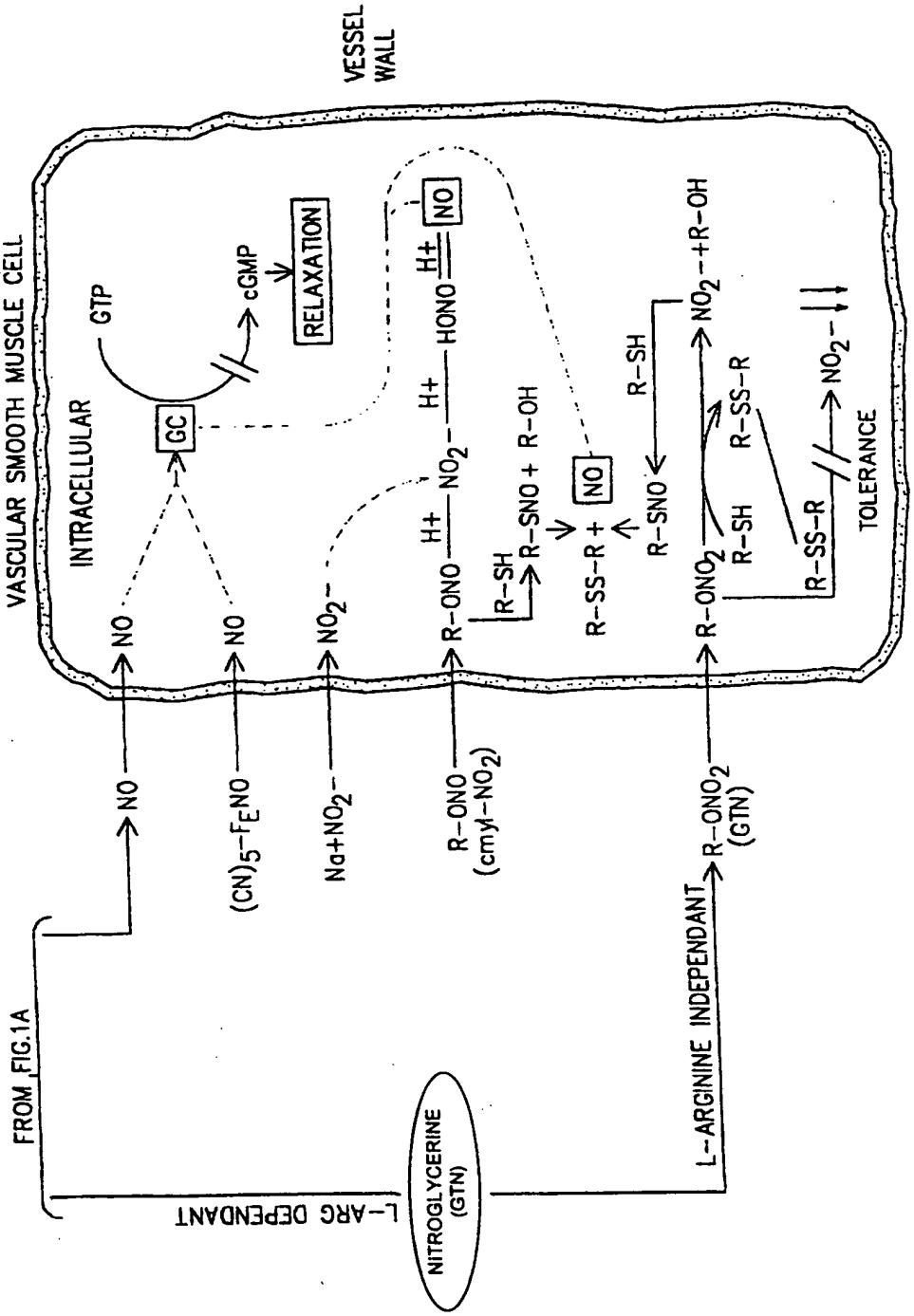
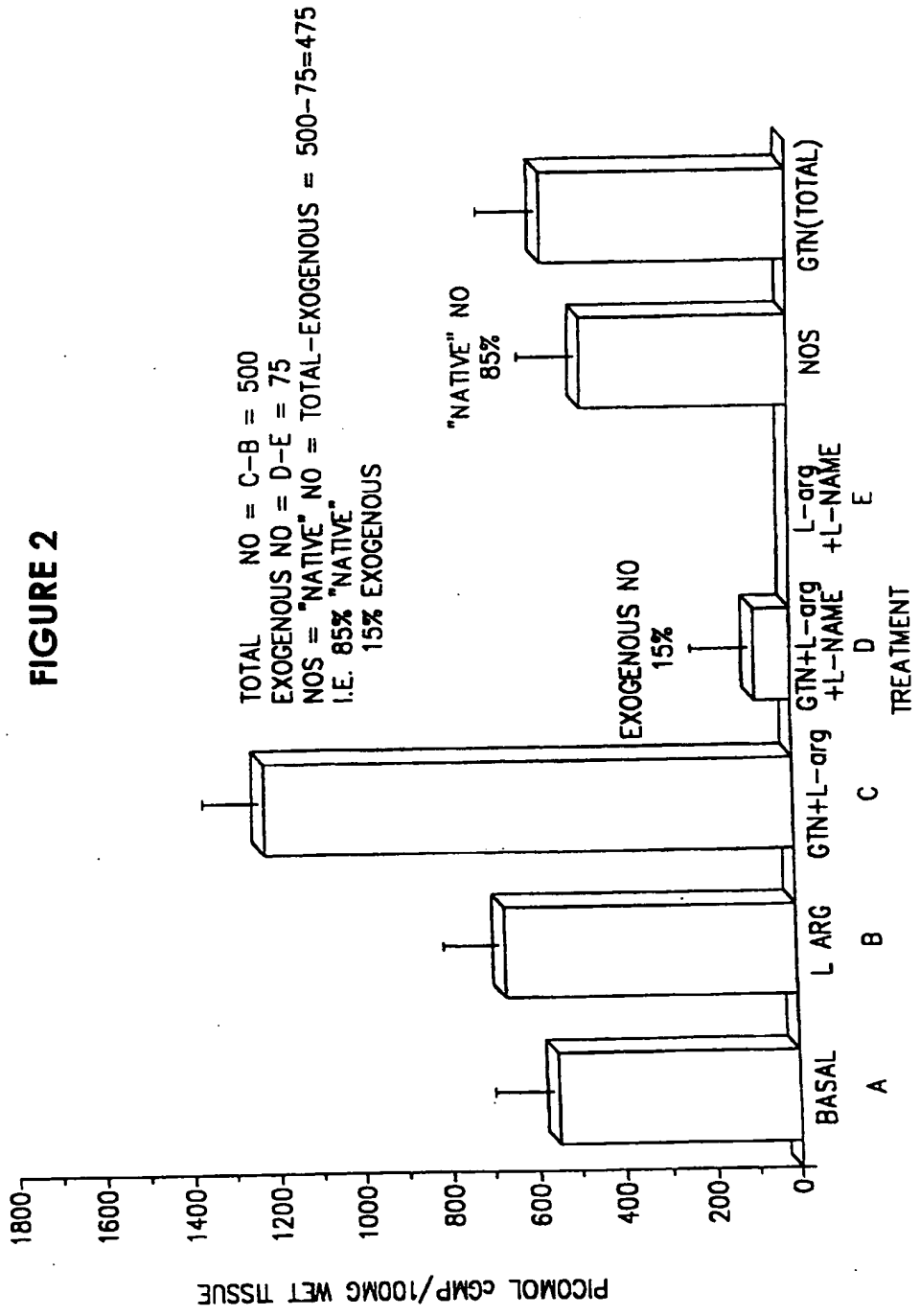

VESSEL WALL
VASCULAR SMOOTH MUSCLE CELL
INTRACELLULAR
GTP
GC
cGMP
RELAXATION
NO
$(CN)_5-Fe^{II}NO$
NO_2^-
R-ONO
H+
NO_2^-
HONO
H+
NO
R-SH
R-SNO + R-OH
R-SS-R +
NO
R-SNO
R-SH
NO_2^- + R-OH
R-ONO$_2$
R-SH
R-SS-R
R-SS-R
NO_2^-
TOLERANCE
NO
$Na+NO_2^-$
R-ONO
(cmyl-NO_2)
R-ONO$_2$
(GTN)
FROM FIG.1A
L-ARG DEPENDANT
NITROGLYCERINE (GTN)
L-ARGININE INDEPENDANT

FIGURE 2


TOTAL NO = C-B = 500
EXOGENOUS NO = D-E = 75
NOS = "NATIVE" NO = TOTAL-EXOGENOUS = 500-75=475
I.E. 85% "NATIVE"
15% EXOGENOUS
"NATIVE" NO
85%
EXOGENOUS NO
15%
PICOMOL cGMP/100MG WET TISSUE
1800
1600
1400
1200
1000
800
600
400
200
0
BASAL
L ARG
GTN+L-arg
GTN+L-arg +L-NAME
L-arg +L-NAME
NOS
GTN(TOTAL)
A
B
C
D
E
TREATMENT

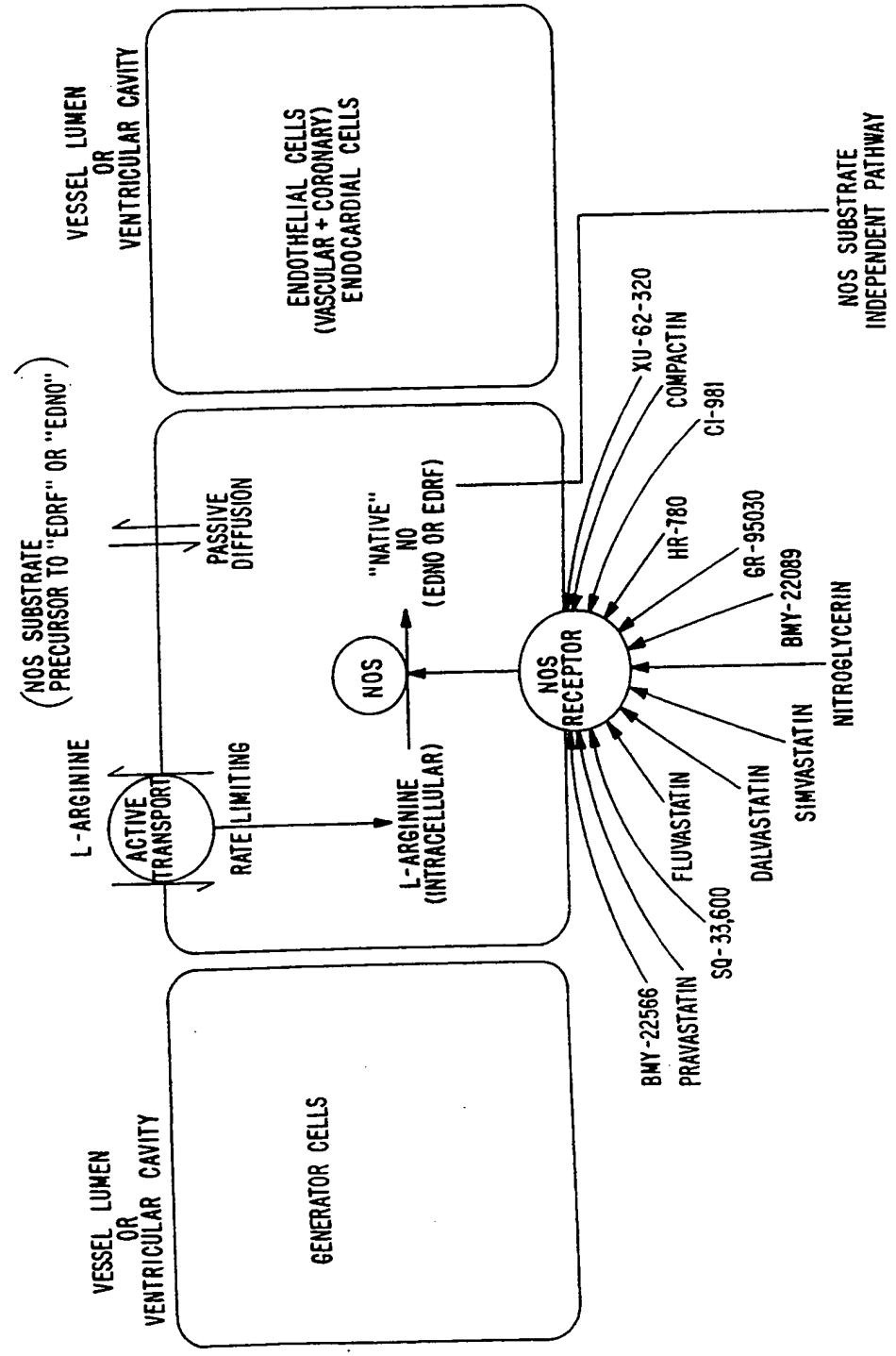

VESSEL LUMEN OR VENTRICULAR CAVITY
ENDOTHELIAL CELLS (VASCULAR + CORONARY) ENDOCARDIAL CELLS
NOS SUBSTRATE INDEPENDENT PATHWAY
(NOS SUBSTRATE PRECURSOR TO "EDRF" OR "EDNO")
PASSIVE DIFFUSION
"NATIVE" NO (EDNO OR EDRF)
L-ARGININE
ACTIVE TRANSPORT
RATE LIMITING
L-ARGININE (INTRACELLULAR)
NOS
NOS RECEPTOR
XU-62-320
COMPACTIN
CI-981
HR-780
GR-95030
BMY-22089
NITROGLYCERIN
SIMVASTATIN
DALVASTATIN
FLUVASTATIN
SQ-33,600
PRAVASTATIN
BMY-22566
VESSEL LUMEN OR VENTRICULAR CAVITY
GENERATOR CELLS

FIGURE 3

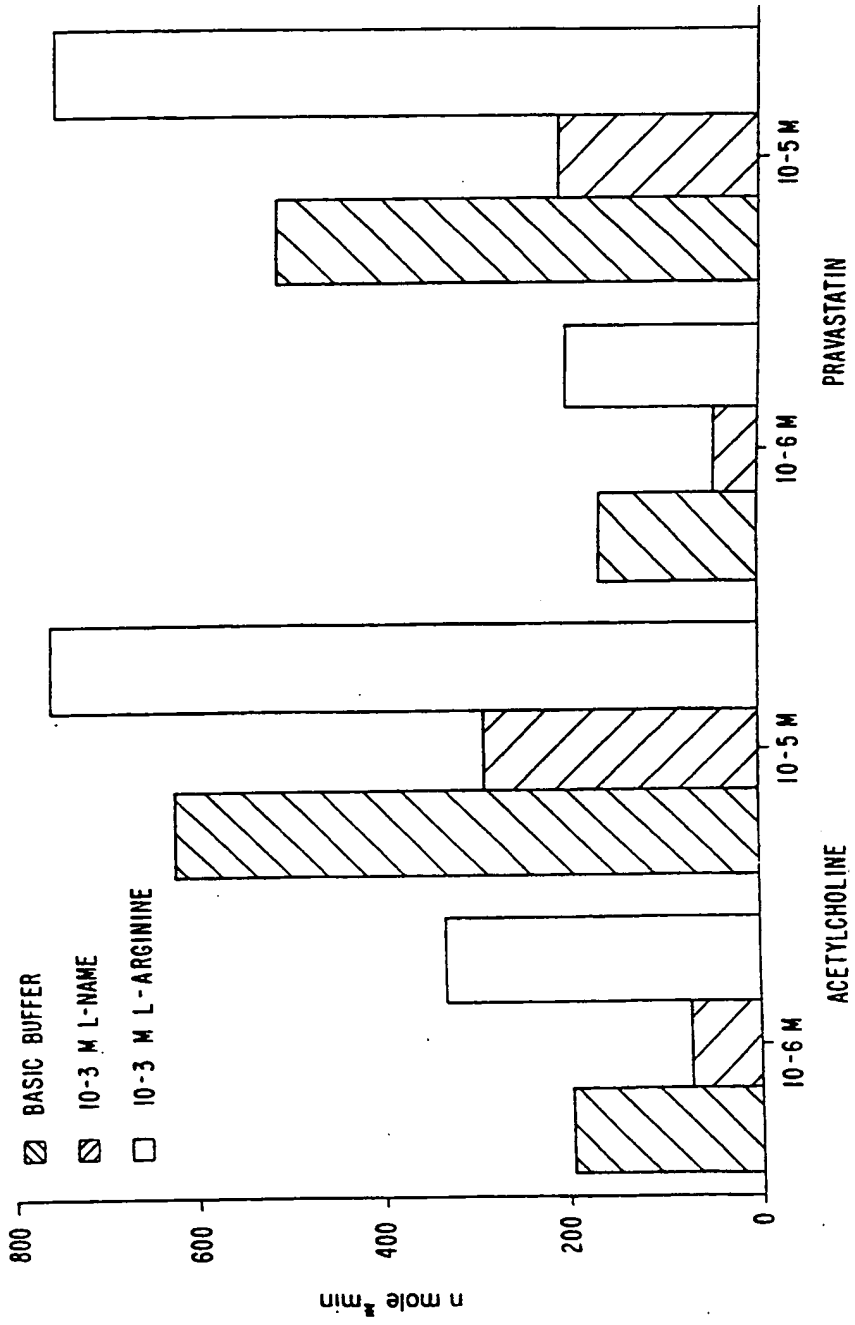

n mole NO/min
800
600
400
200
0
BASIC BUFFER
10-3 M L-NAME
10-3 M L-ARGININE
10-6 M
10-5 M
ACETYLCHOLINE
10-6 M
10-5 M
PRAVASTATIN

FIGURE 4

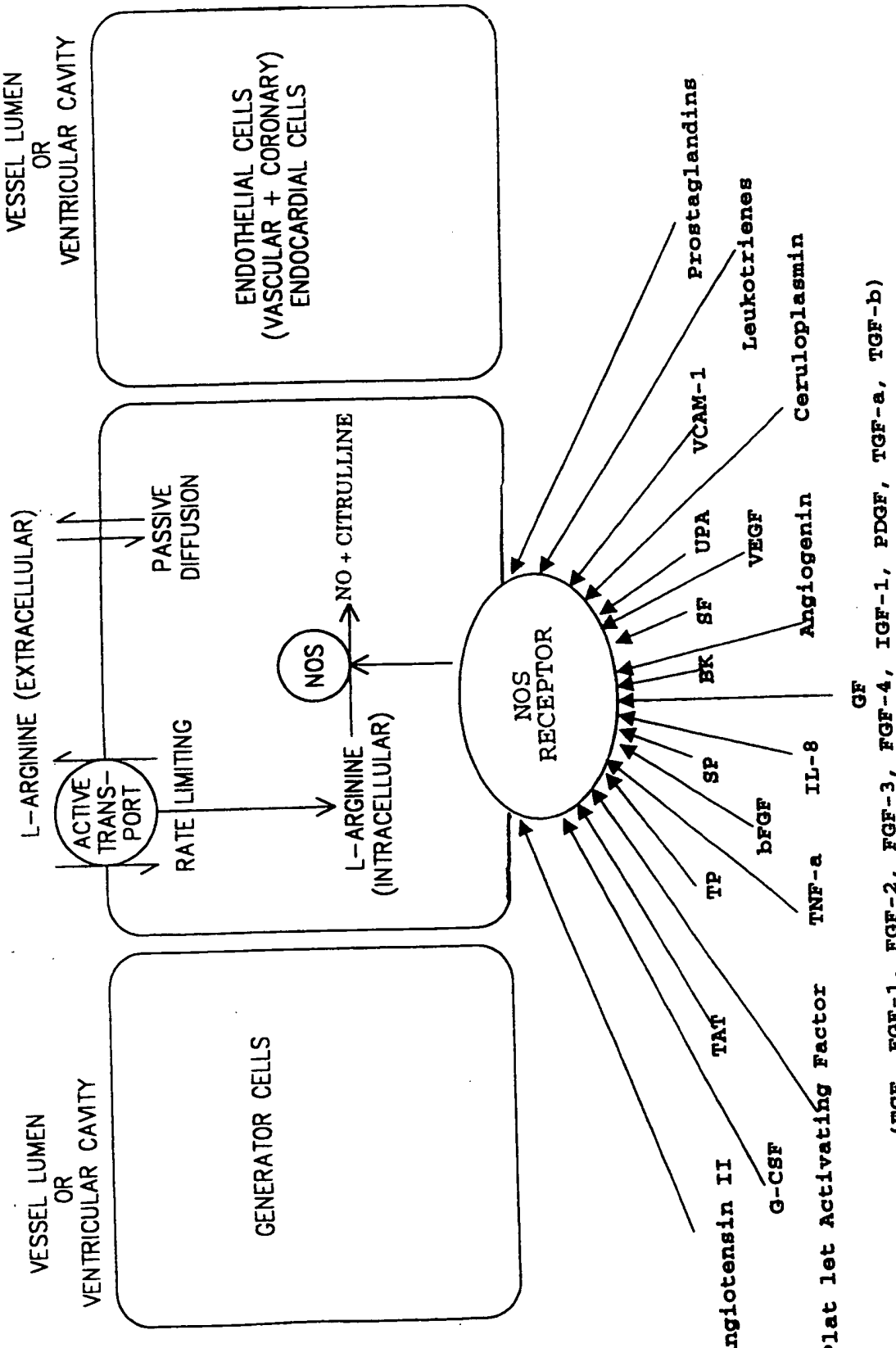

VESSEL LUMEN OR VENTRICULAR CAVITY
ENDOTHELIAL CELLS (VASCULAR + CORONARY) ENDOCARDIAL CELLS
L-ARGININE (EXTRACELLULAR)
ACTIVE TRANS-PORT
RATE LIMITING
PASSIVE DIFFUSION
L-ARGININE (INTRACELLULAR)
NOS
NO + CITRULLINE
NOS RECEPTOR
GENERATOR CELLS
VESSEL LUMEN OR VENTRICULAR CAVITY
Prostaglandins
Leukotrienes
VCAM-1
Ceruloplasmin
UPA
VEGF
SF
Angiogenin
BK
GF
IL-8
SP
bFGF
TNF-a
TP
TAT
G-CSF
Angiotensin II
Plat let Activating Factor
(EGF, FGF-1, FGF-2, FGF-3, FGF-4, IGF-1, PDGF, TGF-a, TGF-b)

FIGURE 5

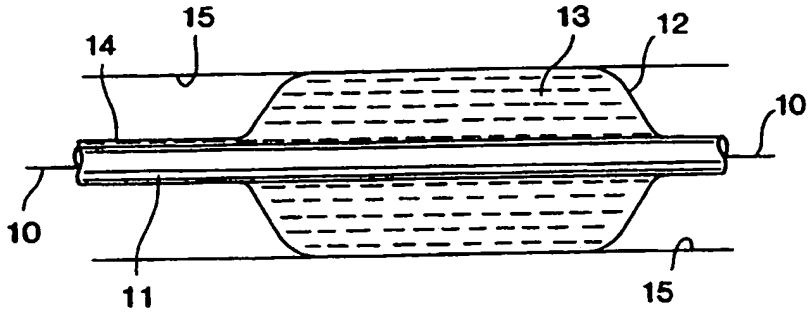


FIGURE 6

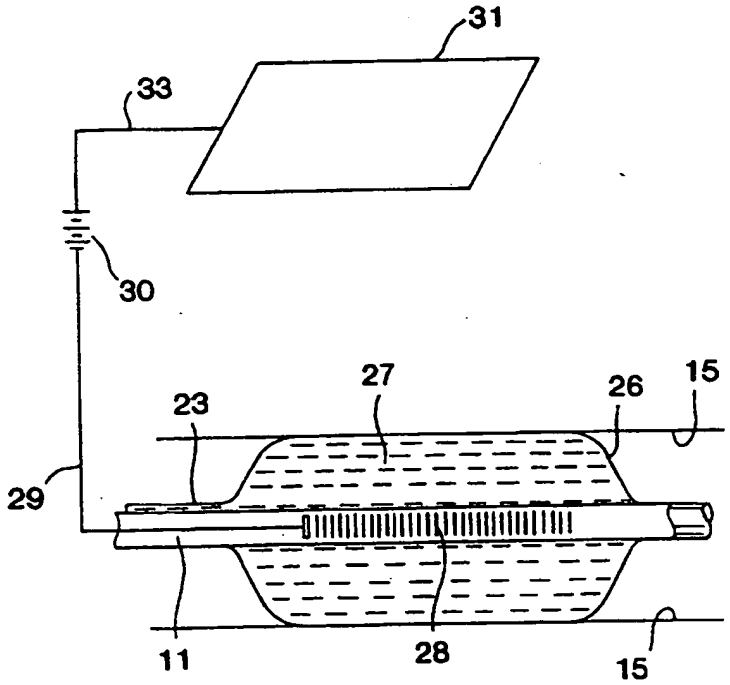


FIGURE 7

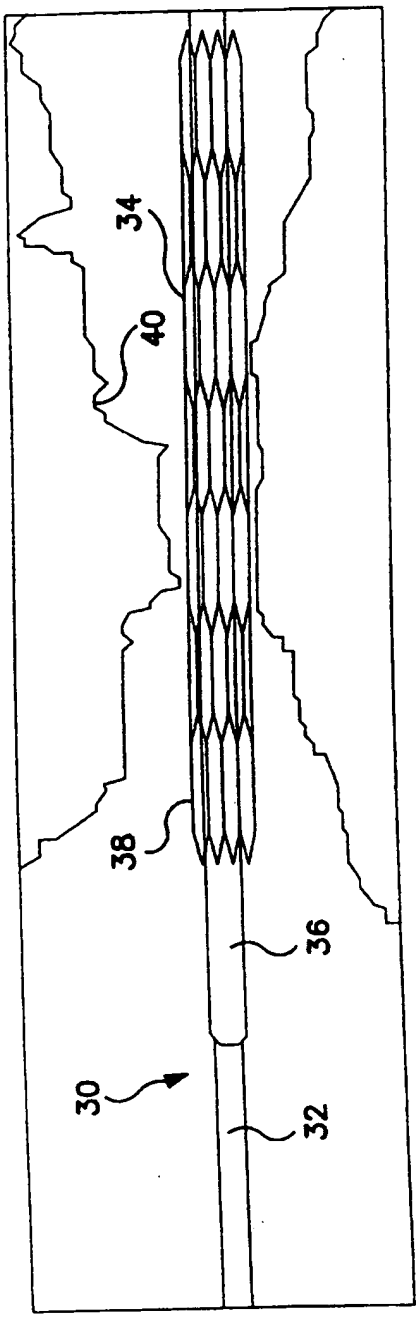


FIGURE 8

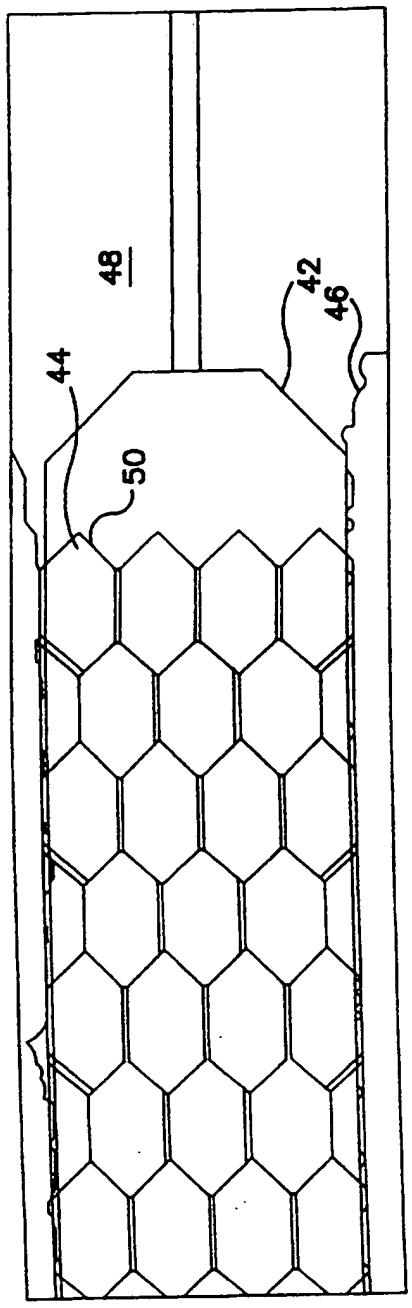


FIGURE 9

FIGURE 10

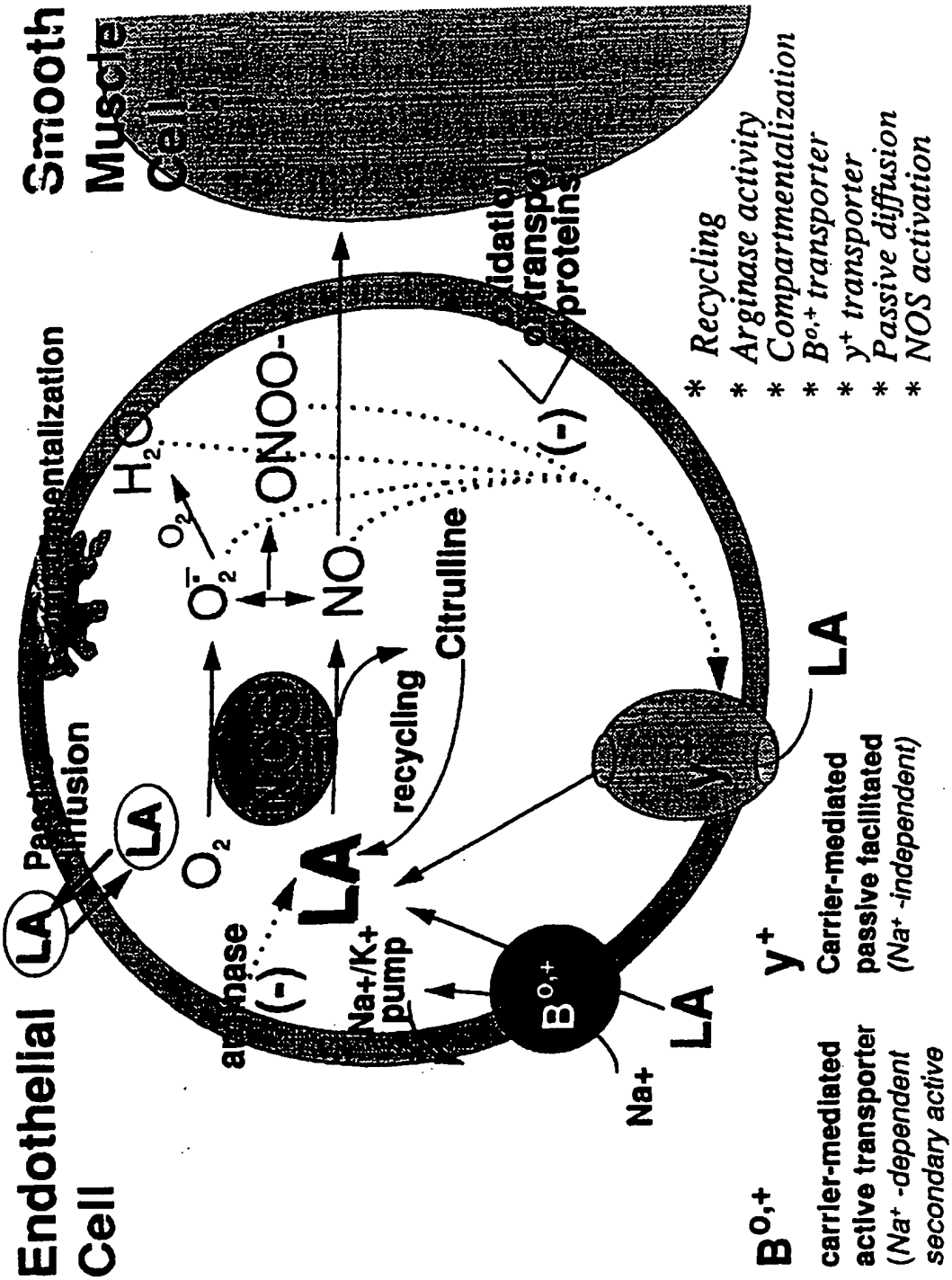

Endothelial Cell
Smooth Muscle Cell
LA
Passive diffusion
Compartmentalization
O_2
O_2^-
H_2O_2
ONOO-
NO
Citrulline
recycling
LA
Na+/K+ pump
(-)
LA
$B^{o,+}$
Na+
LA
$B^{o,+}$
carrier-mediated active transporter (Na^+ -dependent secondary active
y^+
Carrier-mediated passive facilitated (Na^+ -independent)
LA
(-)
oxidation of transport proteins
* Recycling
* * Arginase activity
* * * Compartmentalization
* * * * $B^{o,+}$ transporter
* * * * y^+ transporter
* * * Passive diffusion
* * NOS activation

FIGURE 11

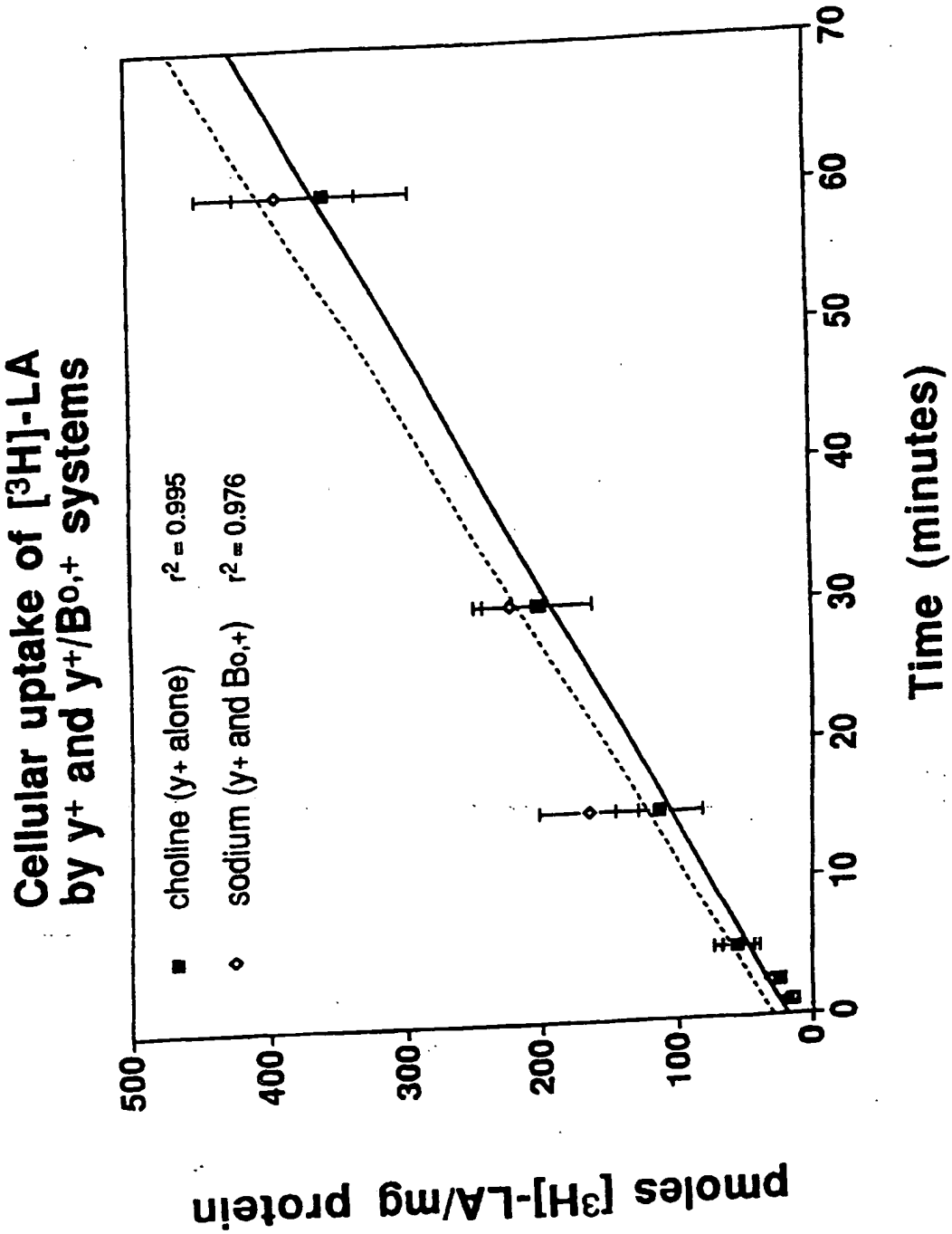

Cellular uptake of [3H]-LA
by y+ and y+/Bo,+ systems
choline (y+ alone) r2 = 0.995
sodium (y+ and Bo,+) r2 = 0.976
pmoles [3H]-LA/mg protein
Time (minutes)
0
100
200
300
400
500
0
10
20
30
40
50
60
70

FIGURE 12

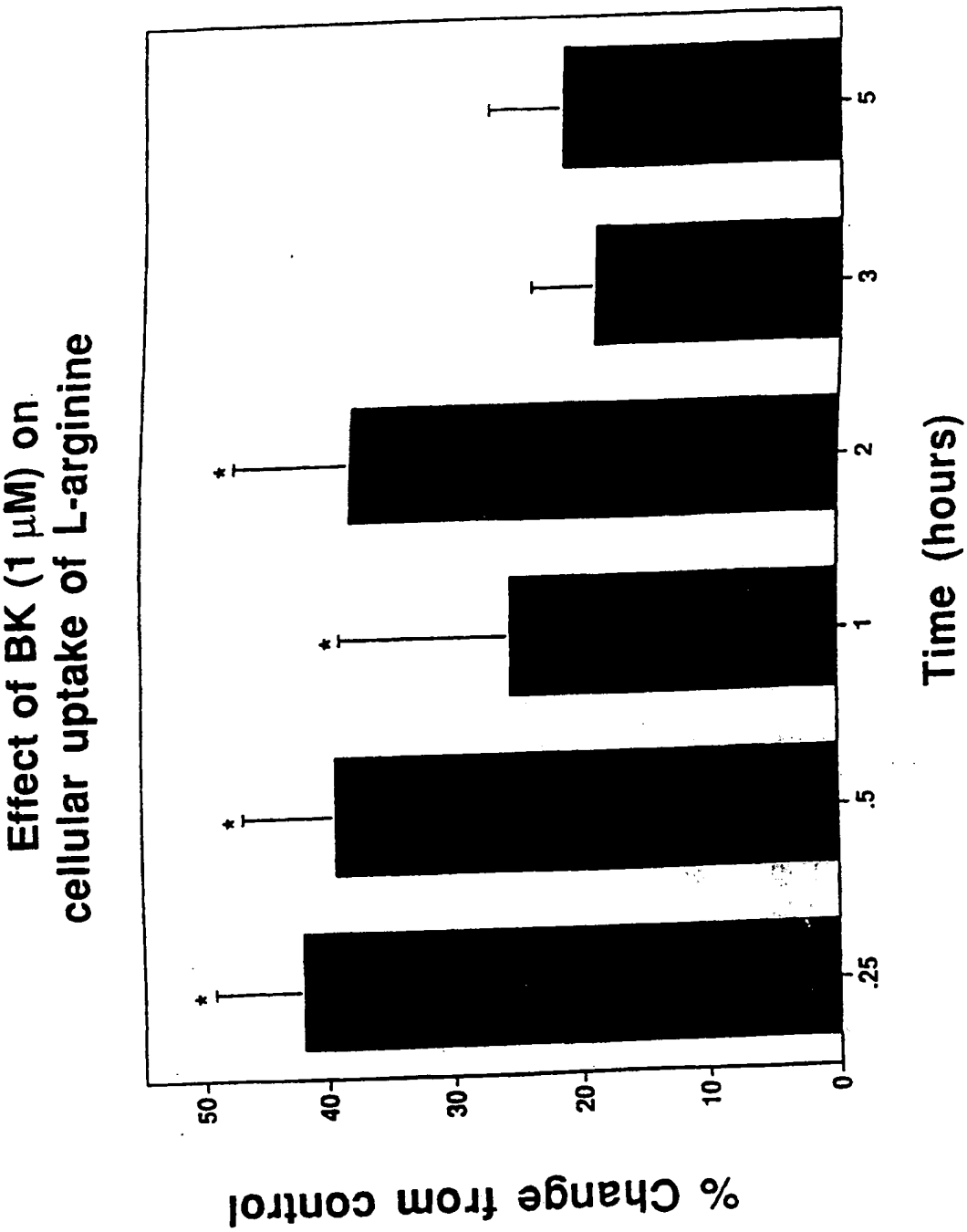

Effect of BK (1 μM) on
cellular uptake of L-arginine
% Change from control
0
10
20
30
40
50
.25
.5
1
2
3
5
Time (hours)
*
*
*
*

FIGURE 13

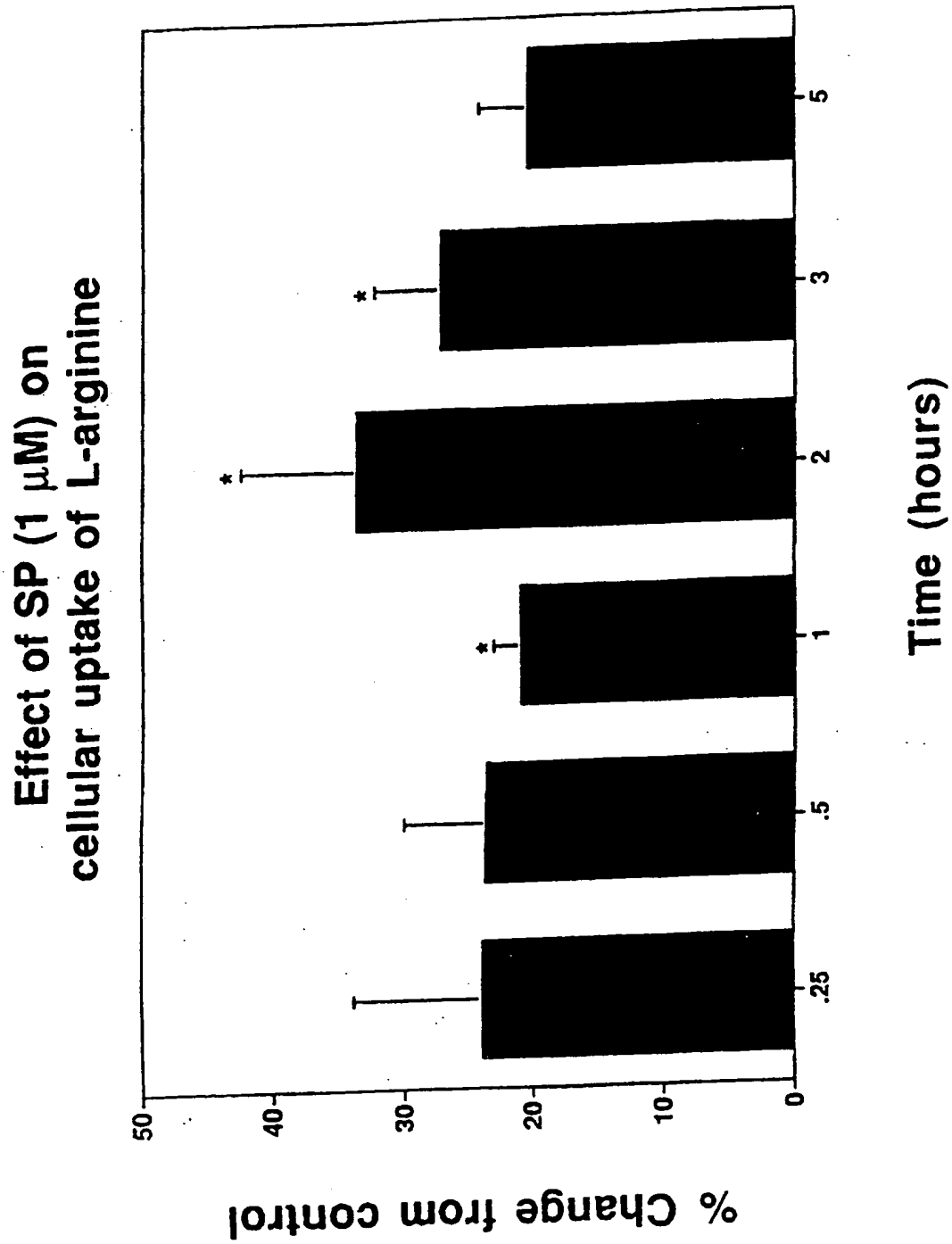

Effect of SP (1 μM) on
cellular uptake of L-arginine
% Change from control
0
10
20
30
40
50
.25
.5
1
2
3
5
Time (hours)
*
*
*

FIGURE 14

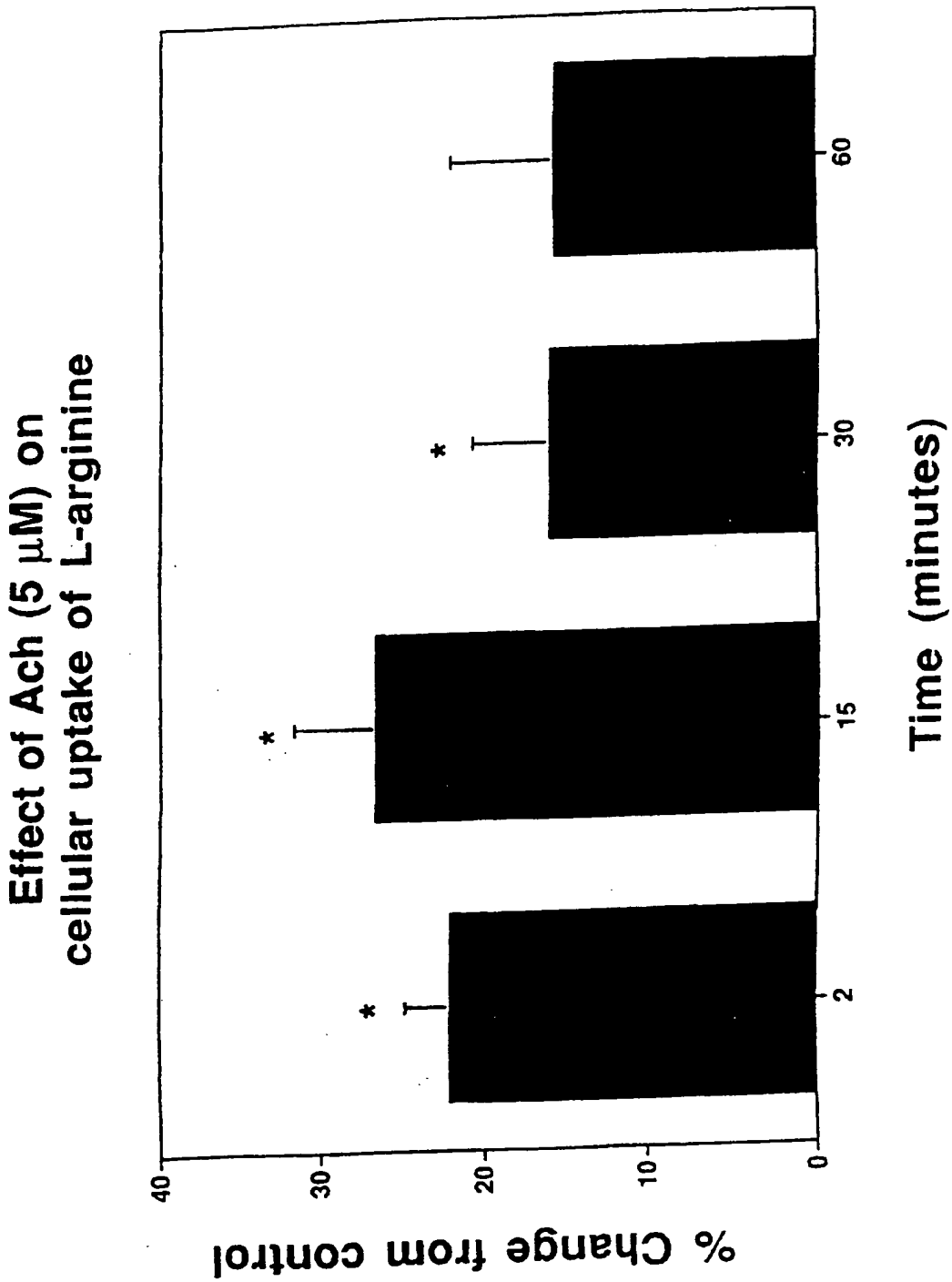

Effect of Ach (5 µM) on
cellular uptake of L-arginine
% Change from control
0
10
20
30
40
2
15
30
60
Time (minutes)
*
*
*

FIGURE 15

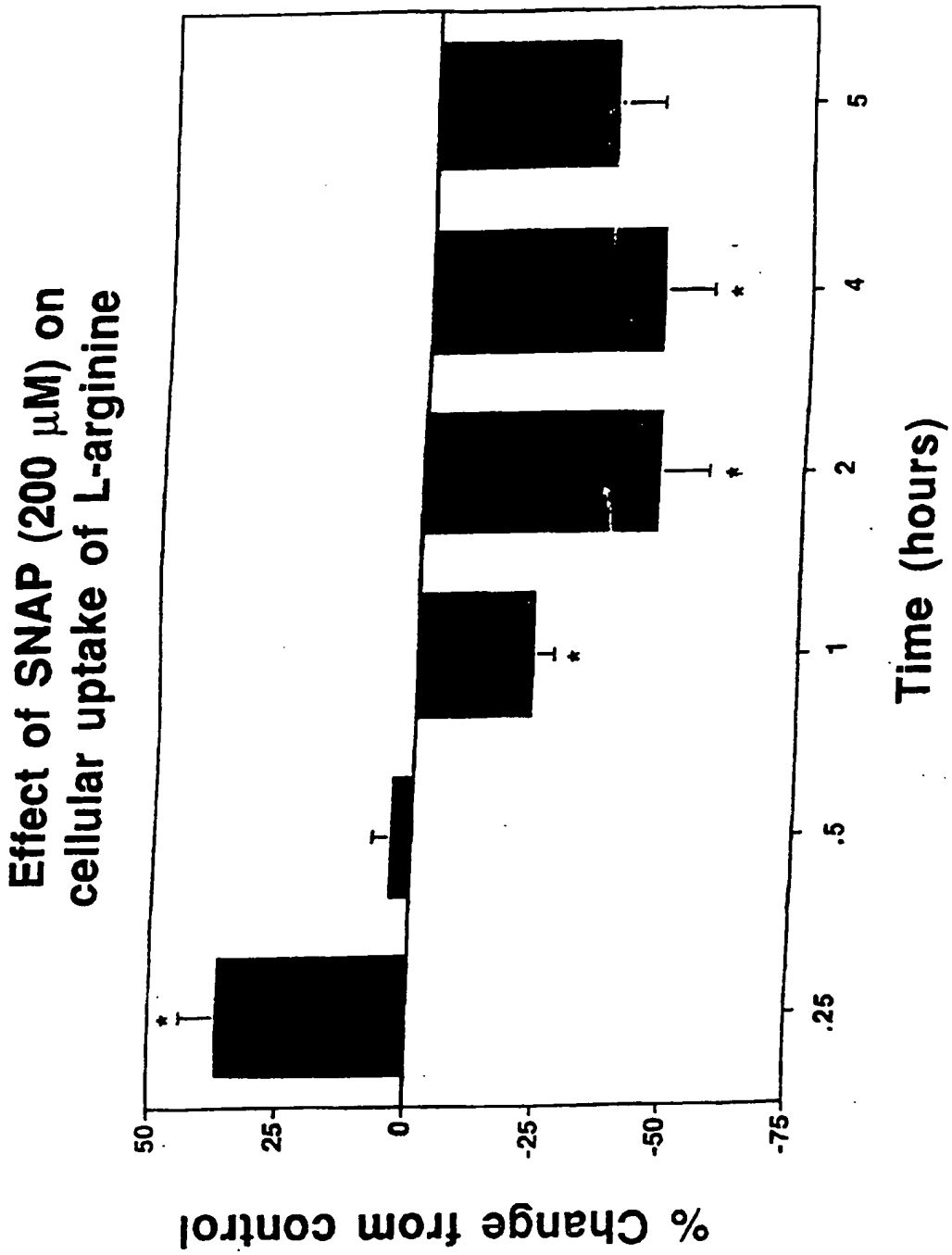

Effect of SNAP (200 μM) on cellular uptake of L-arginine
% Change from control
50
25
0
-25
-50
-75
.25
.5
1
2
4
5
Time (hours)
*

FIGURE 16

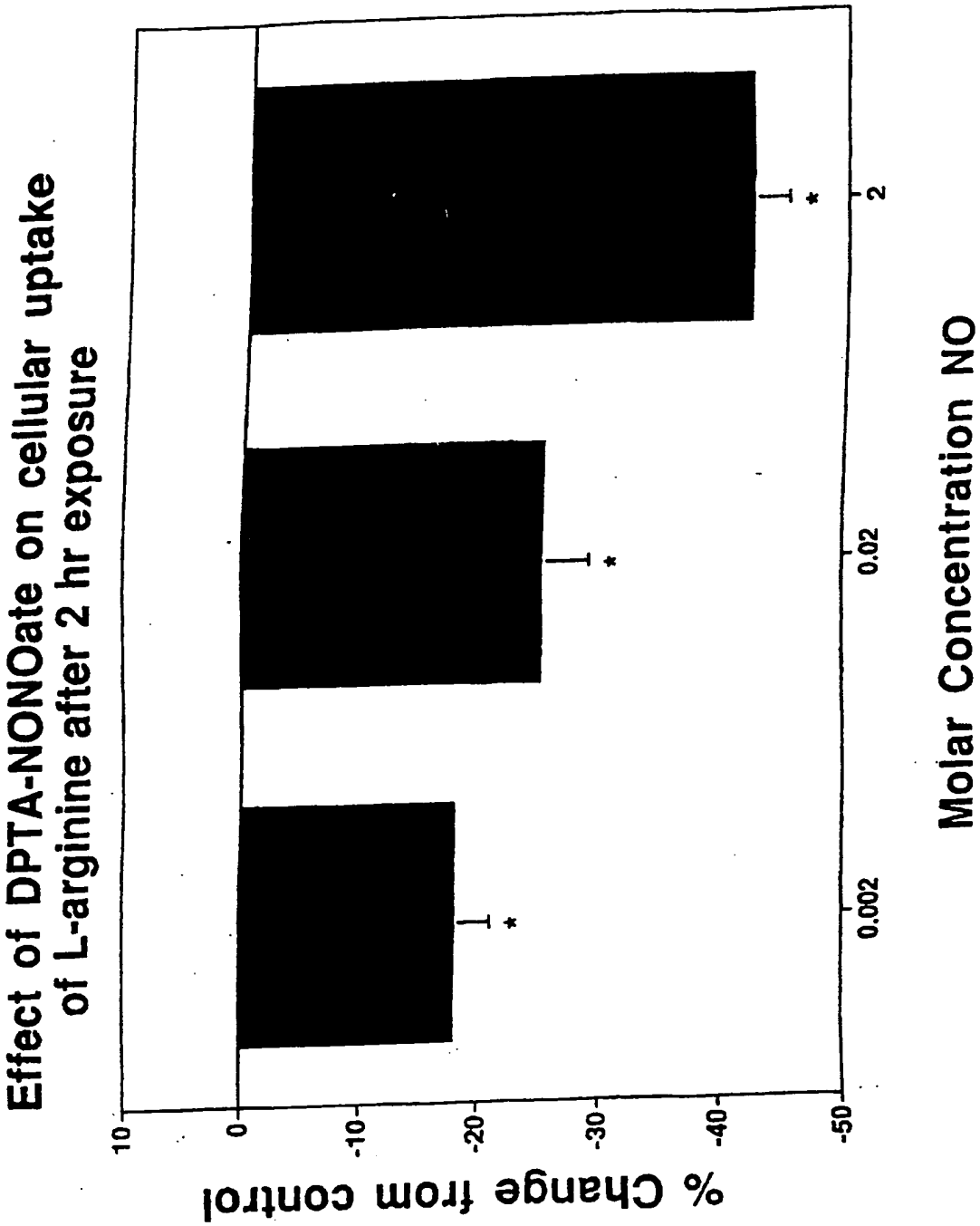
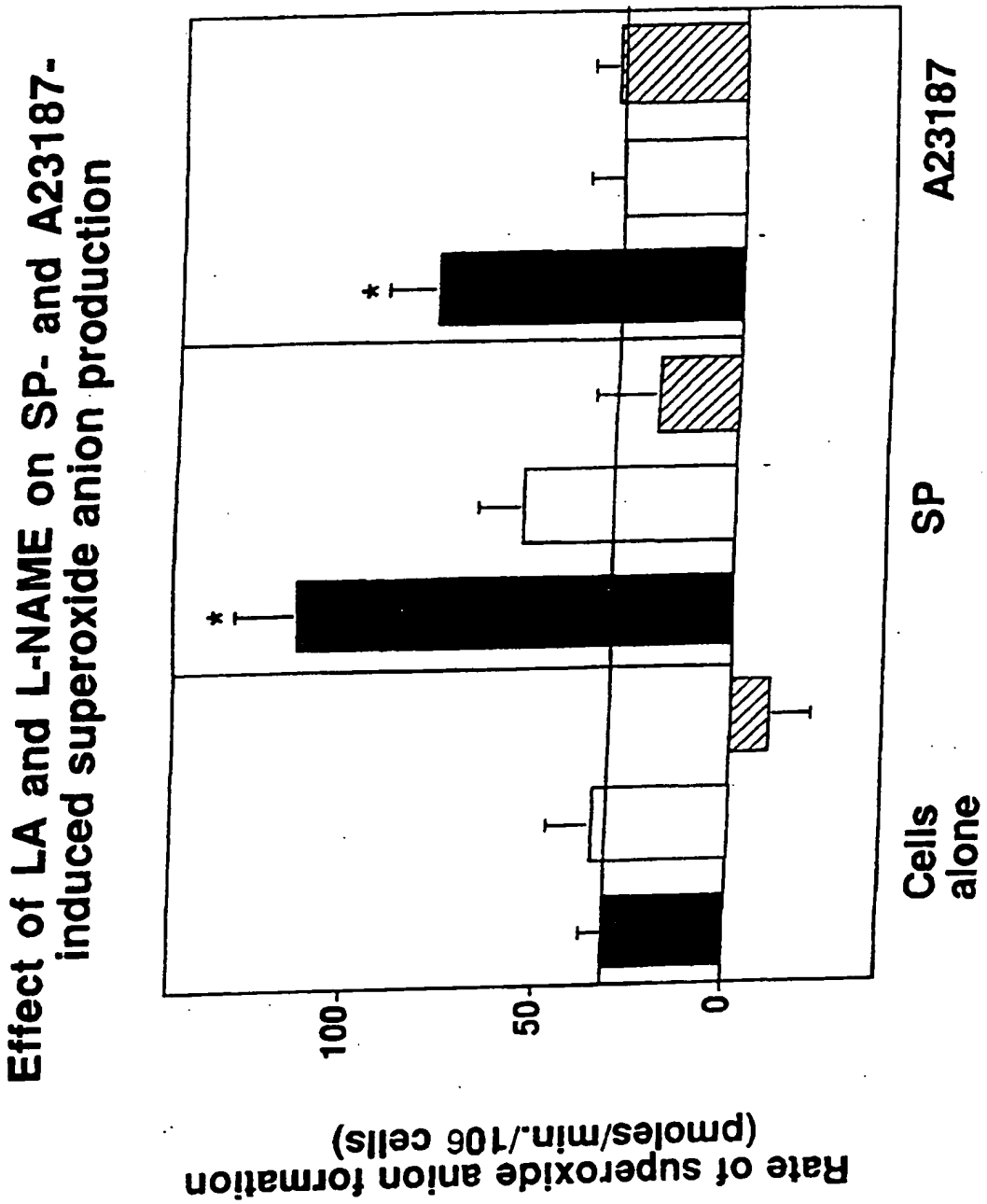

Effect of DPTA-NONOate on cellular uptake of L-arginine after 2 hr exposure
% Change from control
10
0
-10
-20
-30
-40
-50
*
*
*
0.002
0.02
2
Molar Concentration NO

FIGURE 17


Effect of LA and L-NAME on SP- and A23187-induced superoxide anion production
Rate of superoxide anion formation (pmoles/min./10⁶ cells)
100
50
0
*
*
Cells alone
SP
A23187

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/10298

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A01N 37/12, 37/44; A61K 31/195
US CL : 514/564, 565, 460

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/564, 565, 460

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,595,970 A (GARFIELD et al.) 21 January 1997, see abstract and entire document.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search 17 AUGUST 2000	Date of mailing of the international search report 19 SEP 2000
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